

Lipid accumulation, macrophage activation, and liver dysfunction following spinal
cord injury

Honors Research Thesis

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Abstract

The spinal cord innervates all peripheral organs and, accordingly, spinal cord injury (SCI) has a chronic negative effect on peripheral organ function. As such, SCI patients have many chronic medical conditions that reduce health, life span, and quality of life. Metabolic syndrome is one such condition with high prevalence in the SCI population. The liver is essential for regulation of metabolism, and pathological changes in the liver contribute directly to developing metabolic syndrome. Surprisingly, the potential for SCI to disrupt liver functions has not been thoroughly examined. Therefore, we tested the hypothesis that SCI causes chronic liver pathology and dysfunction. Adult female rats were used as a model organism for spinal cord injury. Animals were perfused at different time points following SCI and tissue samples were collected for histological and molecular analyses. Adipose tissue showed changed expression of the adipokines resistin and adiponectin that modify inflammatory and metabolic function after injury. Significant lipid accumulation in the liver occurred by 1 day post injury (dpi) and remained elevated for at least 21 dpi compared to non-injured control animals. Detailed lipidomic analysis revealed that the increased lipid content is coupled with changes in the proportions of specific cytotoxic ceramide species. Coincident with the alterations in lipid content, inflammation increased acutely in the liver and remained elevated through 21 dpi. Inflammatory changes included hepatic macrophage activation from increased expression of interleukin inflammatory factors and TNF- α . Liver dysfunction is similar in rats receiving cervical or mid-thoracic SCI, suggesting these same effects likely occur in a large percentage of the SCI population. Given the liver's essential role in metabolic function and the known metabolic problems after SCI, understanding the extent and cause of liver dysfunction following SCI may identify novel therapeutic targets for improving health, longevity, and quality of life for SCI patients.

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Introduction

Affecting over 270,000 people with 12,000 new cases every year³¹, spinal cord injury (SCI) is a chronic condition affecting the lives of many Americans. As survival rates improve from the initial spinal cord trauma, the deleterious effects of various secondary pathologies are becoming better known¹. The spinal cord innervates all peripheral organs and, accordingly, SCI has been shown to have a chronic negative effect on peripheral organ function. As such, SCI patients have many chronic medical conditions that reduce health, life span, and quality of life³¹. Metabolic syndrome is one such condition with high prevalence in the SCI population⁴. The liver is essential for regulation of metabolism, and pathological changes in the liver contribute directly to developing metabolic syndrome, which is associated with cardiovascular impairment, peripheral inflammation, and increased risk of death⁹. While some studies have aimed to elucidate the changes in the liver following SCI, the role of spinal cord injury level on degree of dysfunction has not been examined. The present study seeks to determine the cause and extent of liver dysfunction following spinal cord injury at different levels of trauma, as well as to further characterize the array of metabolic and inflammatory changes after injury.

Adipose tissue has been indicted in both metabolic and inflammatory changes. Adipocytes were once considered storage vessels for lipids, but have now been recognized for their important endocrine functionality^{15,22}. Adipose tissue is capable of regulating both metabolic and inflammatory changes. Because of these roles, adipose tissue is considered to be the most prominent factor in systemic inflammation^{22,32}. In cases of obesity and high percentage body fat, adipose tissue can become dysfunctional and release excessive signaling factors that lead to inflammation and insulin resistance. Endocrine proteins released by adipocytes are referred to as adipokines, the most prominent being resistin, leptin, and adiponectin. Resistin and leptin are pro-inflammatory adipokines usually present in lower concentrations in the serum. Leptin is highly involved in metabolic function, specifically in monitoring satiety and hunger. Resistin is involved in regulation of metabolism through modifying blood glucose concentration, and also acts in an inflammatory manner itself and by increasing expression of other pro-inflammatory cytokines. Adiponectin acts to counteract many of the effects produced by resistin and leptin. It has been shown to counteract the symptoms of metabolic dysfunction and reduce systemic inflammation. The described functions of adipose tissue and adipokines indicate change to their expression after spinal cord injury could be involved in metabolic dysfunction.

Adipose tissue is certainly a key player in metabolic function, but the most important metabolic organ of the body must play a role in any dysfunction as well. Liver dysfunction can lead to an array of physiological changes that can be

manifested in a variety of detrimental ways. One of the most common liver diseases is non-alcoholic fatty liver disease (NAFLD). NAFLD is a common hepatic disorder of the developed world, affecting 10-24% of the population of various countries³⁰. Due to the high incidence of metabolic syndrome and insulin resistance after SCI, the incidence is likely even higher in the SCI population. High levels of lipids within the liver of individuals without viral infection or excessive alcohol consumption characterize this pathology, of which the pathogenesis is not completely understood. Current models of pathophysiology describe a “multiple-hit” model for disease development¹⁷. The model describes four factors that can lead to NAFLD – increased white adipose tissue and adipokine release, insulin resistance, oxidative stress, and immune activation. Any coincidence of these factors puts an individual at higher risk for NAFLD. When paired with increased hepatic inflammation, the disease progresses to a more advanced condition known as NASH (non-alcoholic steatohepatitis). The steatohepatitis pathology is concerning because it can lead to liver cirrhosis, and is linked to cardiovascular issues and metabolic syndrome^{18,23}. Spinal cord injury induces a complex cascade of changes after injury that could activate any of the four potential “hits” in the development of NAFLD. Any metabolic changes due to spinal cord injury are best investigated by examining these two organ systems and their interactions. By determining the changes that occur in adipose tissue and the liver after injury, novel targets can be identified for therapeutic intervention in the SCI-patient population.

Background

The initial mechanical trauma from SCI is devastating to proper somatic and autonomic function of the nervous system. The damage severs connections between the central nervous system and its peripheral targets, and causes neuron death and decay. This dysfunction is only compounded when secondary effects disrupt the healing process. Neuroglia and immune cells respond to the sudden change by forming a barrier around the injury epicenter known as the glial scar⁴⁴. While the glial scar formation is part of the body’s innate response to allow for proper healing, the scar formation is more detrimental than beneficial in spinal cord injury. The glial and immune cells that form the scar prevent axonal regrowth through the injury site. The inhibition of axonal regrowth combined with neuron death and decay from the initial trauma causes physiological functions that are regulated by the now damaged nervous system to run independently. Maintenance of homeostasis may persist without autonomic control from the nervous system, but any imbalance could be enough to shift the equilibrium of a system to dysfunction. This dysfunction can be the result of the many physiological imbalances created after spinal cord injury for the connections of the immune system, adipose tissue, and the liver¹⁸.

The liver is the main metabolic organ of the body with autonomic innervation that mostly originates from thoracic vertebra 7 cord level (T7) to T12⁴³. The liver is subject to CNS control through direct sympathetic connections from the spinal cord as well as descending parasympathetic pathways. The autonomic control of liver activity can be adversely affected by damage to any neurons involved in the pathway by spinal cord injury. This damage can be further compounded by dysfunction in other systems that act in the liver, such as the immune system. Spinal cord injury has previously been shown to release leukocytes and inflammatory cytokines into circulation within hours after the initial trauma^{8,24}. As these cytokines and immune cells matriculate through the liver, dysfunction is exacerbated by increased inflammation. In addition to the changes occurring in the liver, fat cells housed in adipose tissue respond to the trauma as part of the acute phase of the innate response^{12,20}. Fat cells mobilize free fatty acids (FFAs) by hydrolysis of stored triglycerides for delivery to the liver. All these changes happen within hours after the initial trauma. Under normal physiological circumstances, these changes result in preparation of the body for a reparative/protective response. However, with injury to the regulatory functions provided by the nervous system, damage can accrue and persist chronically. Normally, these FFAs are converted into glucose to power the physiological machinery needed to react to the

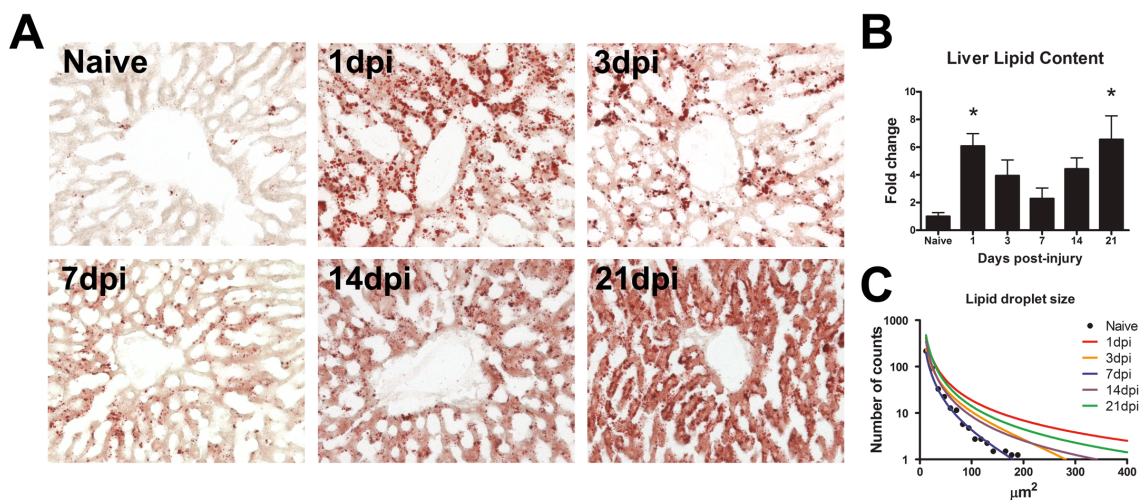


Figure 1.1: Lipid accumulation in the liver is significant as soon as 1 dpi. The increase in lipids marked by Oil Red O staining in (A) mimics the pattern followed by inflammation where intermediate time points trend towards an increase but are not statistically significant in (B). The size of these lipid droplets changes also after injury as shown in (C).

mechanical injury²⁵. However, in the case of spinal cord injury, the excess of FFAs may be too great for complete conversion to glucose or export from the liver. As such, excess lipids accumulate in the liver as soon as 1 dpi as shown in Fig 1.1. The lipid content and droplet size, both indicators of liver pathology, are increased after injury. This histology is consistent with NAFLD, and its rapid onset after SCI indicates the trauma and its after-effects are sufficient to induce dysfunction. While

only 10-25% of NAFLD cases progress to NASH^{6,23}, enhanced liver inflammation is already present after SCI, indicating a more rapid progression to NASH-like pathology.

This chronic persistence of pathological changes to the liver can be observed as early as a few hours after injury. The inflammatory state of the liver changes rapidly after injury and stays elevated out to 21 dpi. The increased inflammation state from SCI leads to prolonged activation of Kupffer cells, macrophages localized to the liver, as evidenced by increased CD68-positive staining within liver tissue as shown in Fig 1.2 (A) and (B) at 21 dpi. Changes to the expression profile of the CD68

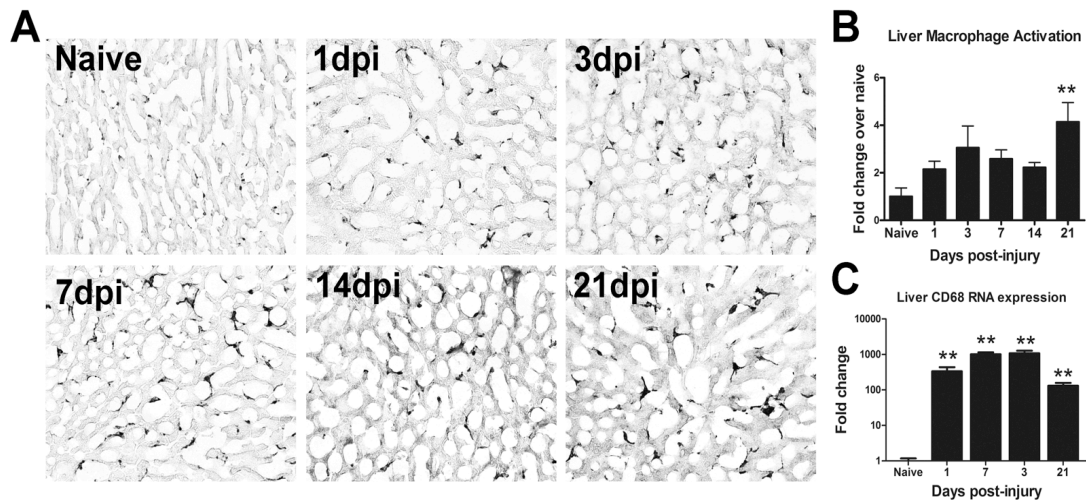


Fig 1.2: The increased inflammatory state of the liver is evident in tissue staining and gene expression. CD68-positive macrophages are stained black in liver tissue in (A) and quantified in (B). Gene expression of CD68 is also increased as soon as 1 dpi and significantly elevated out to 21 dpi as evidenced by RT-PCR data in (C).

gene occur in a more rapid time course. CD68 expression levels are increased as soon as 1 dpi and stay elevated out to the chronic 21 dpi time point. The chronic liver inflammation observed is pathological in nature, and further confirms development of liver dysfunction following spinal cord injury. The steatosis of liver cells paired with the chronic inflammation is concerning because of the damage to hepatocytes and liver function. Both histological markers are present for NASH, and liver damage due to this pathophysiology has been confirmed by serum assays that assess liver function.

The liver pathology is confirmed from increased serum levels of alanine-transaminase (ALT), a clinical marker of liver pathology. ALT is produced in abundant levels within liver cells, and this enzyme leaks into the blood serum in cases of hepatocyte damage due to increased membrane permeability. As shown in Fig 1.3, serum content of ALT is significantly increased at 21 dpi – the same point at which liver lipid accumulation and inflammation reaches significance. The increase

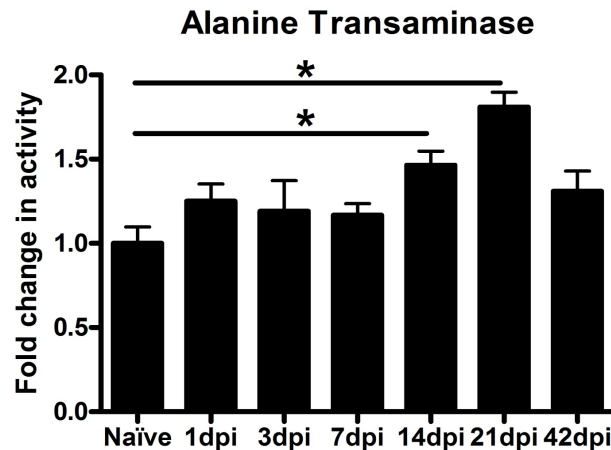


Figure 1.3: Serum levels of alanine transaminase (ALT) are increased significantly at 14 and 21 dpi, further confirming the pathology associated with inflammation and lipid accumulation.

in serum ALT following injury is clearly worrisome for SCI patients developing this pathology. Many non-SCI patients that are diagnosed with this condition are able to use dietary and activity changes to combat this pathology; improved nutritional intake and exercise have been shown to reverse some effects of NAFLD/NASH¹⁷. However, this is more concerning for the SCI patient population because of the mobility restrictions and loss of regulatory control from the spinal cord trauma. Furthermore, these studies indicate the development of NASH independent of typical causes, which may prevent standard lifestyle interventions from being protective.

Clearly, further studies are needed to determine the extent and causes of these pathological changes in the liver following injury. The previous studies have been useful for identifying the liver issues following SCI – it is clear that injury to the spinal cord has the ability to cause rapid onset of liver lipid accumulation and inflammation. However, this information does not reveal the cause of these changes. The purpose of the present research is to expand on these studies to better understand the changes occurring in the liver. These studies explore the changes after injury that may lead to development of NASH – changes in adipokine expression from adipose tissue, changes in liver lipid composition and expression of inflammatory genes, and the differences in pathology between cervical and thoracic injury models.

Methods

Spinal Cord Injury Methodology

Spinal cord injury was performed using the Sprague-Dawley rat as a clinically relevant model for SCI. All procedures performed on animals were approved for use

by IACUC and conformed to all standards set forth by the NIH and The Ohio State University animal care guidelines. Rats (220-280g; Harlan, Houston, TX) were anesthetized by an intraperitoneal injection of a solution of ketamine (80 mg/kg) and xylazine (10 mg/kg). A dorsal laminectomy was performed at either the cervical-vertebre level 5 (C5) or thoracic-vertebre level 8 (T8). Rats were then injured by the Infinite Horizons device (Precision Systems and Instrumentation) to produce a spinal cord contusion injury with 200 kD of force. Animals were allowed to recover after closing the injury site and subcutaneous administration of 5 mL saline solution.

Gene Expression Analysis

Rats were sacrificed at variable times following injury (1-28 dpi) by intraperitoneal administration of a lethal dose of ketamine (120 mg/kg) and xylazine (15 mg/kg) and transcardially perfused with 0.1M phosphate buffered saline (PBS). The liver, adipose tissue, and spinal cord were removed and immediately homogenized in Trizol (15596018, Life Technologies) for preservation of nucleic acids. Following thorough homogenization, samples were flash frozen in liquid nitrogen and stored at -80 °C until analysis. RNA was purified from samples by differential precipitation and centrifugation by standard chloroform/phenol protocol². Purity was assessed by microdrop spectrophotometry before amplification. RNA was then synthesized into cDNA using random primers and MMLV RNA-dependent DNA-polymerase enzyme to create cDNA. Quantitative Real-Time Polymerase Chain Reaction (Applied Biosystems) was used to examine gene expression of the various genes described throughout the following section. Expression levels of each sample were normalized to 18S gene expression and mRNA quantities calculated by the $\Delta\Delta CT$ methods³⁶.

Tissue Removal and Preparation

Animals were sacrificed at the appropriate time post injury as described above. After the tissue was cleared of blood using 0.1M PBS, animals were perfused with 400 mL of 4% paraformaldehyde (PFA). The cross-linking polymerization reaction of the PFA fixed the tissue in place to stabilize the tissue for analysis. Livers were removed and post-fixed in 4% PFA for 2 hours and then transferred to 0.1M phosphate buffer (PB) overnight. The next day, livers were removed from PB and placed in 30% sucrose solution for 3 days. The livers were then frozen on dry ice and blocked in optimal cutting temperature solution (OCT) to be cut into 10 μ m slices in a cryostat and mounted on slides (Superfrost Plus Slides, Fisher Scientific).

The tissue was cut in OCT at -20 °C and slides were stored at the same temperature until immunohistochemistry (IHC) analysis.

Histological Analysis of Liver Tissue

IHC was performed to analyze macrophage inflammation in liver tissue. CD68 antibody (Serotec, MCA341R, 1:2,000) was used to visualize hepatic macrophages. Oil Red O staining was used for analysis of liver lipid accumulation. Tissue slides were placed in 70% ethanol for 10 min and then removed and placed into saturated Oil Red O solution in 70% ethanol for 30 min at 60 °C. Differentiation in 70% ethanol was the final step before a wash in distilled water ended the reaction. Both IHC and Oil Red O stained sections were dehydrated in ethanol and organic solvent (histoclear) and then covered with glass held by Permount (Thermo Scientific). Quantification of both staining protocols was performed with microscopic imaging using MCID software (Imaging Research Inc) of sections chosen at random in each time point group. CD68 was quantified by positive staining divided by the proportional tissue area. Oil Red O was quantified by positive staining divided by hepatocyte area using MCID quantification.

Blood Serum Analysis

Cardiac blood was collected from animals during perfusion and allowed to clot at room temperature for 30 mins after withdrawal. After centrifugation of clotted blood at 3,000xG for 10 min, supernatant was collected and again centrifuged for 3 min at 3,000xG to isolate blood serum. Serum was frozen at -80 °C until analysis. Serum levels of alanine transaminase were assessed by ELISA assay (Caymen Chemical, cat #700260). The assay was performed following the provided manufacturer's instructions and normalized to a known standard.

Statistical Analysis of Data

Analysis was performed by statistical software (GraphPad Prism, San Diego, CA) using one-way ANOVA tests and post-hoc analysis to determine statistical significance. Minimum level of significance was of a p-value less than 0.05. Outliers were confirmed by the Q-test for statistical outliers.

Results

Expression of adipokines is changed following spinal cord injury

Previous studies shown above have shown the increased inflammation and lipid accumulation in the liver following injury. The present experiments were aimed at determining the presence and extent of these changes. Changes in adipose tissue and expression of adipokines, metabolic signaling factors derived from adipose tissue, were explored to examine changes in the primary fat storage organ. Adiponectin, resistin, and leptin were examined because of their inflammatory and metabolic effects on a target organ^{32,37}. These adipokines act to alter the immune activation already observed after SCI. These changes begin with increased expression of all adipokine genes in visceral and retroperitoneal fat after cervical injury as shown in Fig 2.1. The changes were significant compared to naïve levels for adiponectin and resistin in cervical injuries in visceral fat pads. While the other changes were not significant, resistin and leptin both show increasing transcription trends after both injury level types. Leptin expression is altered the least, as it is not significantly changed in neither visceral nor retroperitoneal fat tissue. Adiponectin

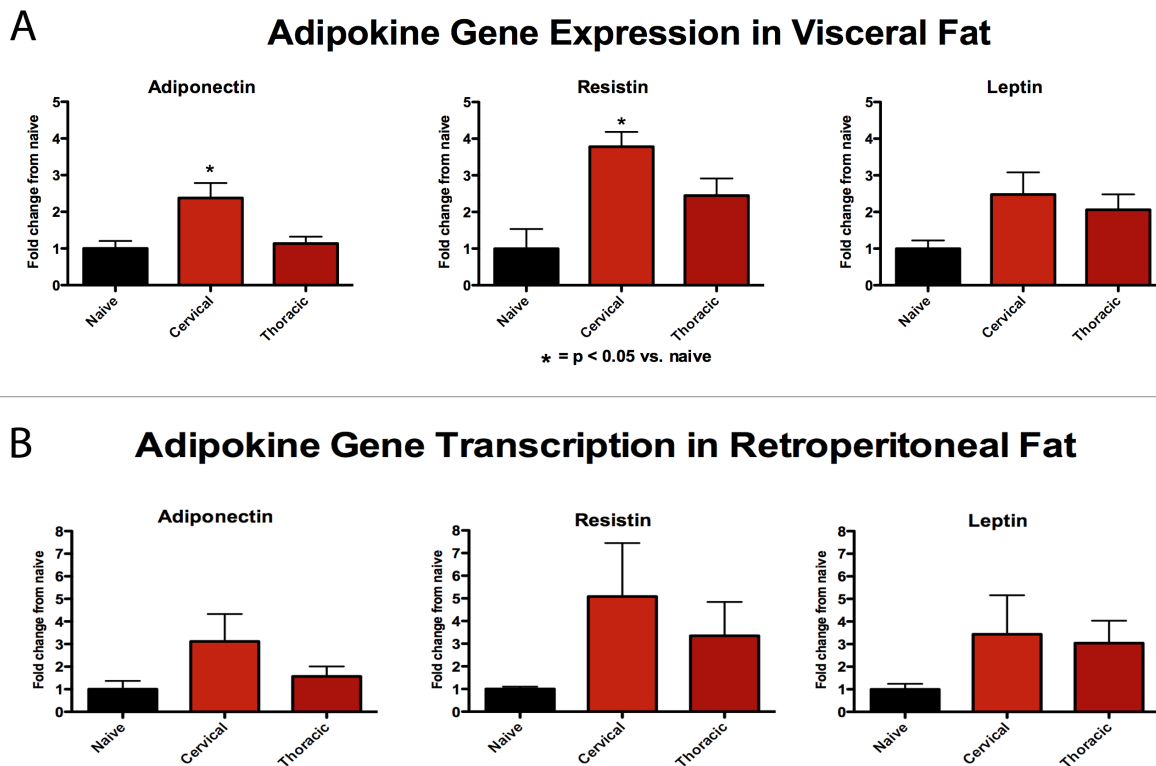
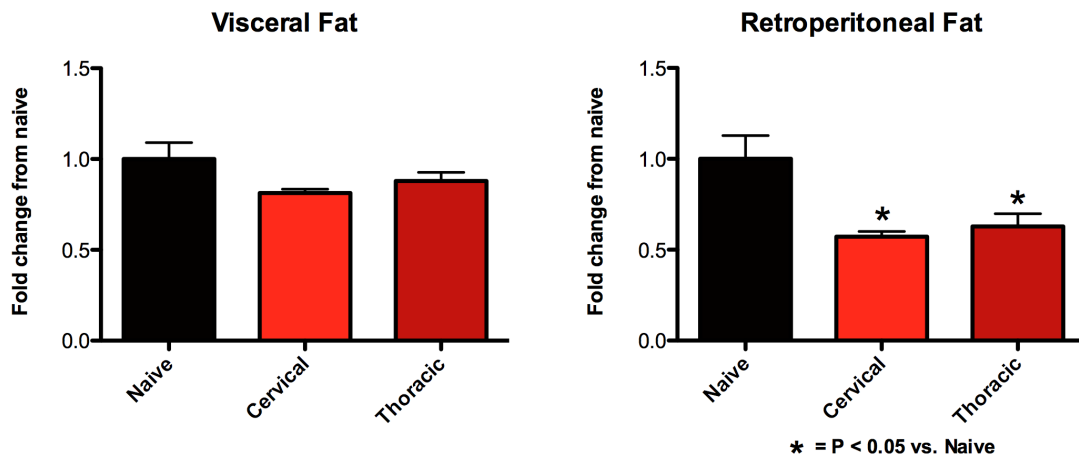


Figure 2.1: The changes in adipokine gene transcription after spinal cord injury at different levels. Changes shown are based off fold changes from naïve tissue after normalization to 18S gene transcription levels. (A) shows the changes of the three adipokines in visceral fat and (B) shows changes in retroperitoneal fat.

is not significantly increased after thoracic injury. These deviations would be expected to reveal similar changes in protein levels in adipose tissue and serum. Western blot analysis revealed that adipose tissue and serum levels of adiponectin

and resistin are different after thoracic injury. Surprisingly, resistin protein levels decrease in retroperitoneal fat while staying unchanged in visceral fat. However, these levels are markedly changed in serum, as adiponectin decreases and resistin increases following injury as shown in Fig 2.2 (B) at 21 dpi following thoracic injury.

A Resistin Protein Quantity in Adipose Tissue



B Serum Adipokine Protein Expression after Thoracic SCI

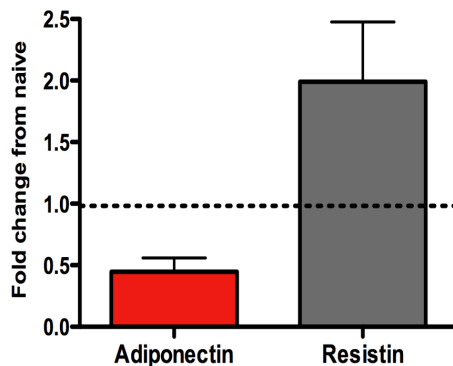
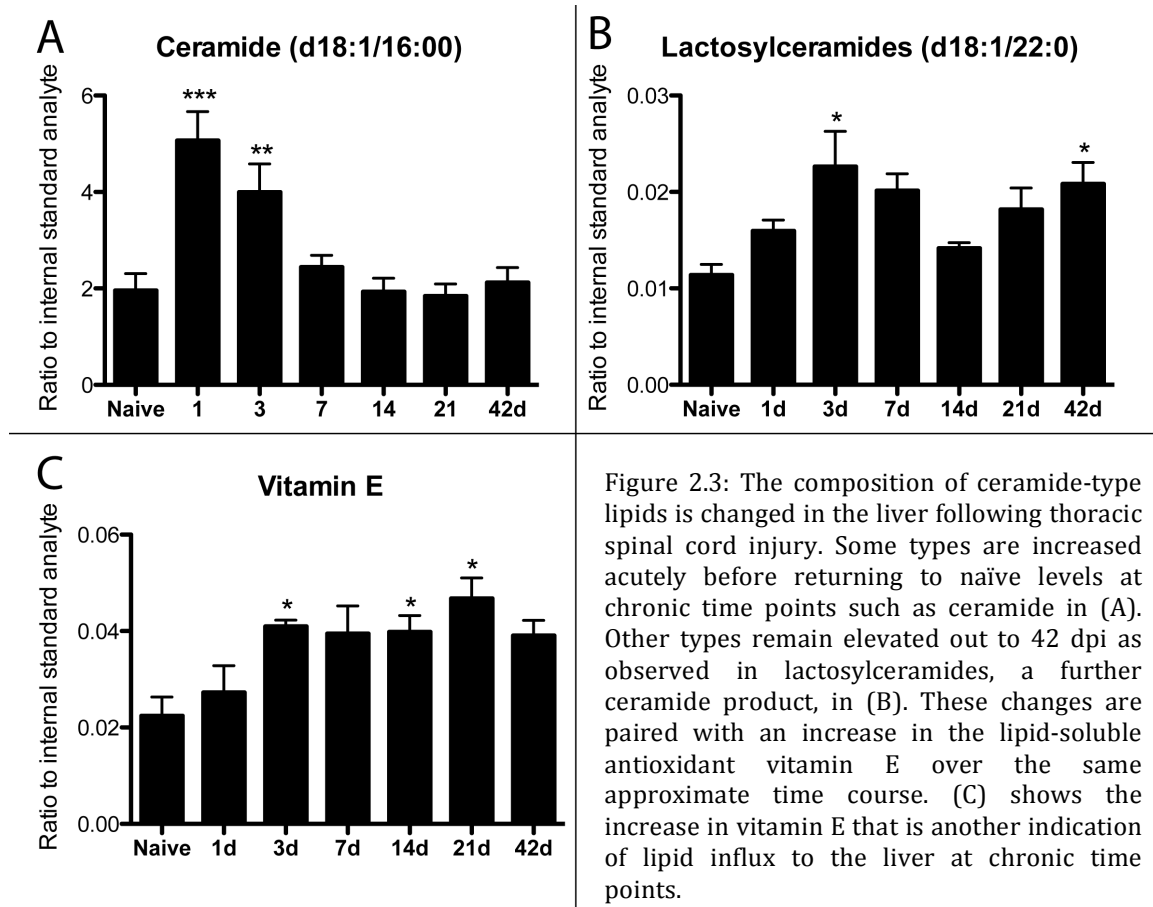


Figure 2.2: Changes in protein expression after different injury levels. (A) shows the changes in resistin expression in adipose tissue. (B) shows the changes in resistin and adiponectin expression in blood serum after thoracic injury. Changes in adiponectin in adipose tissue were not significantly different (data not shown.)

Injury causes a change in hepatic lipid composition

Previous studies have shown the pathological accumulation of hepatic lipids as soon as 1 dpi after injury, but did not investigate the composition of these lipids. Lipidomic analysis of liver tissue was used to determine the type of lipids that were changed in addition to the normal triglyceride increase. It was found that the composition of lipids in the liver was changed after SCI. Cytotoxic ceramide species were found to change in the liver following injury. Different ceramides showed significant increases or decreases. As shown in Fig 2.3, ceramide and

lactosylceramide levels increased acutely after injury, but only lactosylceramide remained elevated up to 42 dpi. These changes are reflective of changes in other ceramide-type lipid species after injury. This is paired with an increase in hepatic levels of the lipid-soluble Vitamin E at 3, 14, and 21 dpi. It was also vital to determine the source of the increased ceramide species to find potential therapeutic targets to prevent hepatocyte damage from ceramide over accumulation.



The origin of the changing landscape of the fat stored in the liver was also explored with extensive gene profiling. Ceramides come from a common precursor (dihydroceramides) that can be derived from de novo synthesis methods within the liver or through salvage pathways using sphingomyelin as a metabolite³³. It was found that the liver contributes to ceramide synthesis from a de novo pathway, as transcription for the rate-limiting enzyme SPT (serine-palmitoyl transferase) was increased at 3 and 21 dpi as shown in Fig 2.4 (A). In contrast, neutral Sphingomyelinase (nSMase), the enzyme responsible for the salvage pathway of ceramide synthesis, was unchanged from naïve levels through 21 dpi in Fig 2.4 (B).

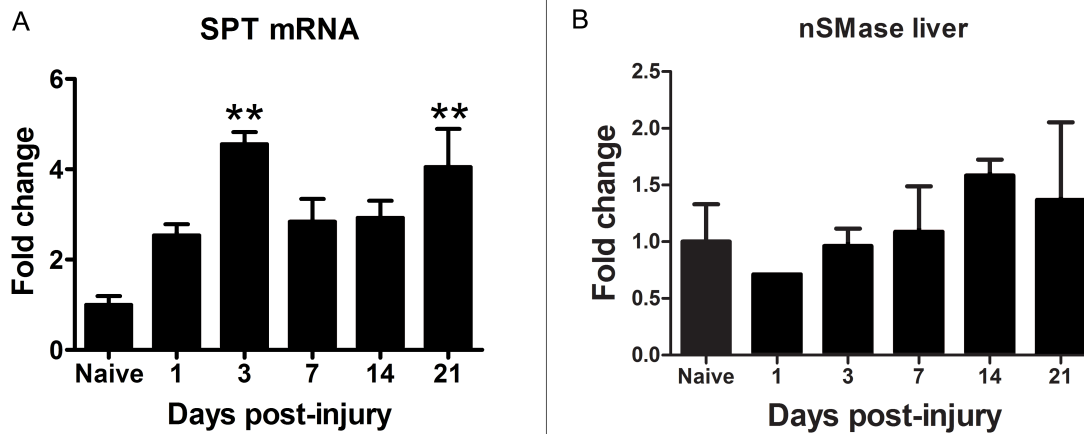


Figure 2.4: Change in RNA expression in liver tissue of rate-limiting enzymes in de novo and salvage pathway synthesis of ceramides. SPT mRNA shown in (A) is the rate limiting enzyme in de novo ceramide synthesis. (B) shows nSMase RNA – the rate limiting enzyme in the salvage pathway that catabolizes sphingomyelin into ceramides.

Inflammatory gene expression is increased following injury

Previous studies have shown an enhanced inflammatory response in the liver acutely following injury as shown above. The increased activation of macrophages housed in the liver results from activation by cytokines, and it was important to determine which cytokines were increased that caused this activation. Therefore, changes in inflammatory genes were assessed out to 3 weeks post-injury. Notably, tumor necrosis factor (TNF- α) is significantly increased at 14 and 21 dpi as shown in Fig 2.5 (A). Interleukin-6 (IL-6) and IL-10 are both cytokines secreted by immune

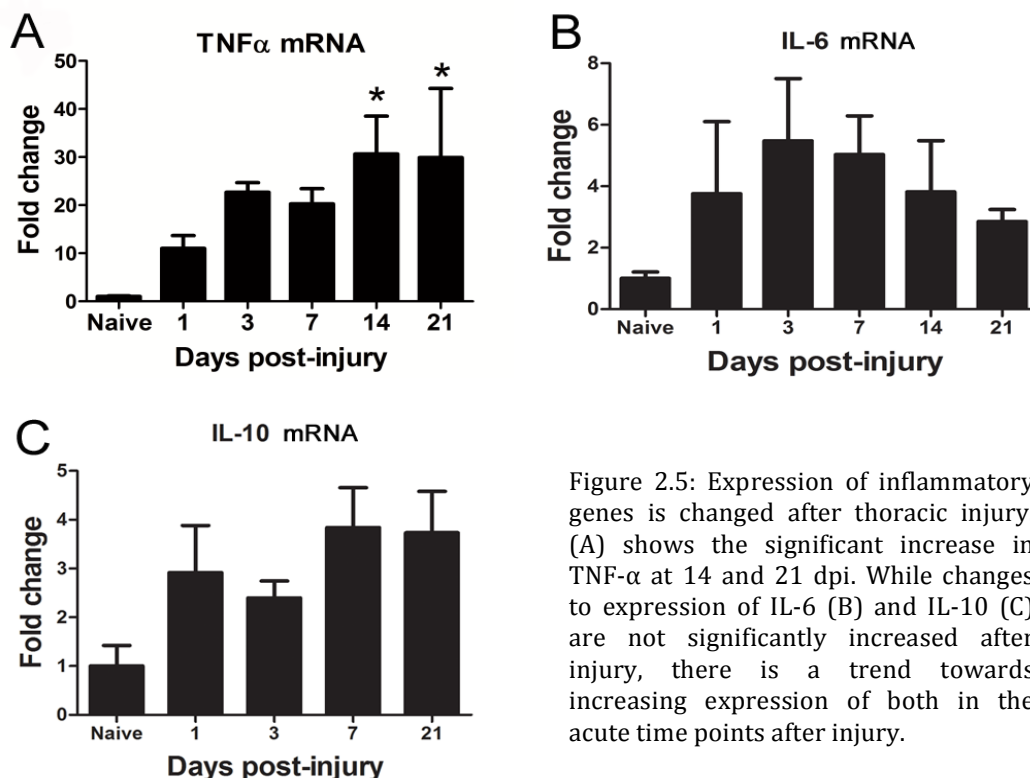


Figure 2.5: Expression of inflammatory genes is changed after thoracic injury. (A) shows the significant increase in TNF- α at 14 and 21 dpi. While changes to expression of IL-6 (B) and IL-10 (C) are not significantly increased after injury, there is a trend towards increasing expression of both in the acute time points after injury.

cells housed in the liver that modulate the inflammatory state of the immune system. IL-6 is a pro-inflammatory cytokine that enhances inflammation, and IL-10 an anti-inflammatory cytokine that acts counteractively to decrease the inflammatory state. Neither cytokine shows significant changes after injury, but both show trending towards increased levels in acute time points.

Cervical spinal cord injury produces comparable liver dysfunction as thoracic spinal cord injury

Fatty liver pathology and increased hepatic inflammation are both observed after thoracic level injury, but a critical aspect of determining the extent of the pathology was to determine their applicability to the heterogeneous injury types of the SCI patient population. The lipid accumulation and inflammation in the liver observed during thoracic injury were also observed in cervical injured animals.

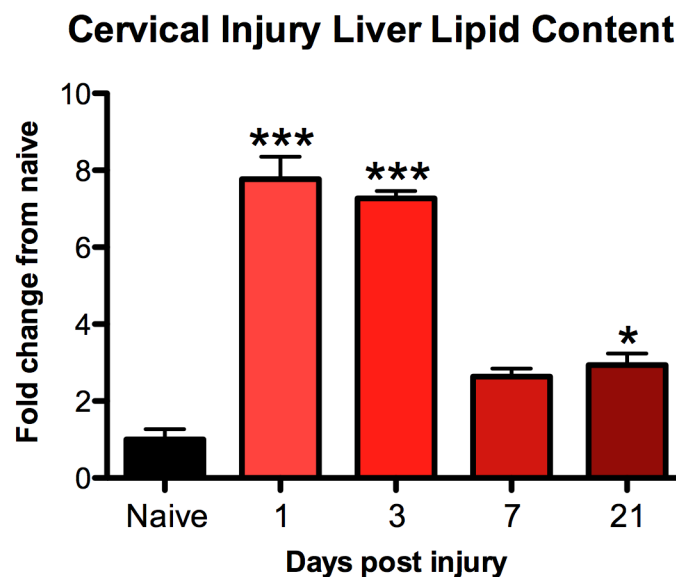


Figure 2.6: Liver lipid content as detected by the Oil Red O stain is increased following C5 cervical spinal cord injury as compared to naïve levels. These changes mirror those observed in thoracic spinal cord injury. 1 and 3 dpi are significantly elevated with $p < 0.01$ compared to naïve content. After no significant change is observed at 7 dpi (similar to thoracic injury), significant increase occurs chronically at 21 dpi with $p < 0.05$ compared to naïve tissue.

Increased liver lipid content was observed as soon as 1 dpi and remains elevated chronically out to 21 dpi. Furthermore, transcription levels of the pro-inflammatory cytokines IL-1 α and IL-1 β both increased chronically following cervical and thoracic spinal cord injury. While these changes are not significant for cervical injury, the increasing trends are evident from the plots shown in Fig 2.7. Unlike the increases in other inflammatory genes, IL-1 α decreased acutely before returning to naïve levels. Confirming the development of liver pathology following cervical injury, serum

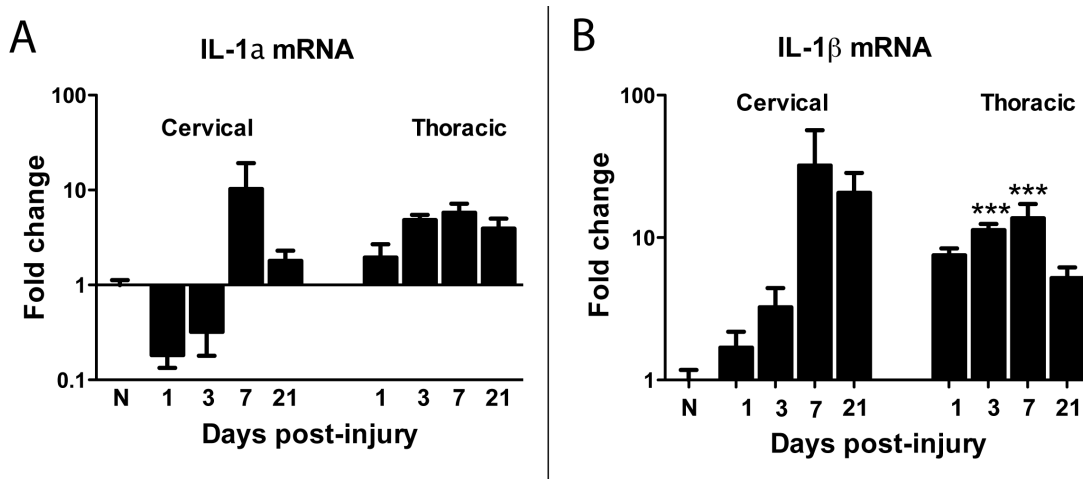


Figure 2.7: Transcription of proinflammatory IL-1 genes following cervical and thoracic injury. (A) shows IL-1 α and (B) shows IL-1 β transcription levels. These follow similar patterns with the exception of the slight decrease of IL-1 α expression acutely after cervical injury.

levels of ALT are increased at 21 dpi as shown in Fig 2.8. The timing of this increase closely resembles the rate of significant serum ALT levels following thoracic injury.

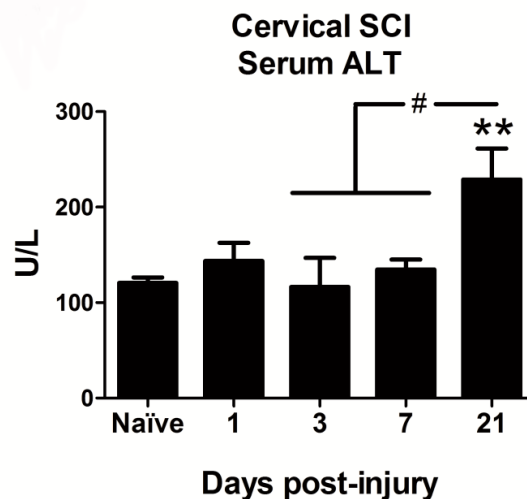


Figure 2.8: Serum levels of alanine transaminase after cervical injury. At 21 dpi, levels of ALT are significantly elevated compared to naïve as well as 3 and 7 dpi. The time course of this increase follows a pattern similar to thoracic injury.

Discussion

The changes invoked by SCI in adipose tissue caused a shift in expression of adipokines, which can produce a myriad of inflammatory changes. Metabolic changes are also enacted in response to these factors, notable enhancement of fat-metabolizing β -oxidation by adiponectin and a decrease in this same fatty acid catabolism reaction when resistin is present²⁸. It is important to consider adipokine levels in both adipose tissue and serum as changes in adipose tissue would alter the metabolic profile of fat tissue, and serum levels would change metabolic effects in

other peripheral organs such as the liver. Metabolic consequences result from altered adipokine expression in both adipose tissue and in the liver. Adipose tissue also plays a key role in initiation of the inflammatory response²². A pro-inflammatory adipokine response can damage the liver and drive chronic liver dysfunction.

From these studies it is clear that adipokine expression is changed after cervical or thoracic injury. However, differences in the direction of gene and protein changes make interpreting these changes somewhat difficult. While leptin does not demonstrate a significant change in expression, both adiponectin and resistin expression is altered following injury in adipose tissue. The present data demonstrates that resistin, a factor indicted in propagating an inflammatory immune response¹⁹, is the adipokine most increased after spinal cord injury. As this pro-inflammatory adipokine migrates to the liver via the blood stream, it would help prolong the inflammatory state caused by the initial injury. This supports previous observations that show chronic liver inflammation out to 42 dpi, and resistin likely prolongs this inflammatory state. Unusually, this increase in gene expression of resistin shown in Fig 2.1 is coupled with a decrease in resistin protein content in adipose tissue as shown in Fig 2.2 (A). This could be explained by the increase in serum resistin content in the same time course. As resistin is produced by adipocytes, most of it is shipped out through blood to aid the immune system by initiating an inflammatory response and to alter the metabolic landscape to provide the organism with fuel to enact these changes. Adiponectin is also increased following cervical injury as shown in Fig 2.1, while it is statistically unchanged during thoracic injury. Adiponectin is anti-inflammatory in nature and helps regulate blood glucose levels by catabolism of lipids via β -oxidation. Adiponectin acts antagonistically to resistin³², and it is interesting to note that the expression of both genes is increased following cervical injury. Despite their antagonistic effects, both adipokines are produced in healthy, uninjured adipose tissue, and their paired increased expression should only serve to initiate the physiological changes needed to respond to traumatic cervical injury. However, in the thoracic injury model, adiponectin was not significantly increased as contrasted to the cervical injury model. It is evident from the presented data that the injury alters the landscape of adipokine expression.

Mobilization of lipids to the liver is one change observed that is connected to the changed expression of adipokines following injury. The decrease in serum adiponectin and increase in serum resistin after 21 dpi provides the strongest evidence that adipose tissue plays a role in pathogenesis of NAFLD/NASH. As serum levels of resistin are increased without the balancing effects of adiponectin, its effect on peripheral organs becomes more pronounced. One effect of this imbalance is insulin resistance⁵. Resistin has been linked to insulin resistance, and adiponectin

has been shown to induce insulin sensitivity^{13,28}. The serum levels shown in Fig 2.2 (B) indicate that insulin resistance can result from this imbalance. As cells become insulin resistant, lipolysis of triglycerides in adipose tissue begins to export FFAs to liver tissue for transformation into glucose. The enhanced levels of serum resistin after thoracic SCI would also allow the liver to remain in a state of chronic inflammation. This could help explain, in part, the chronic inflammation and lipid accumulation of the liver leading to NAFLD as observed in previous and the current studies. However, the specific function of each of these adipokines is still not clearly defined²⁸. Ultimately, more studies are needed to determine the precise changes and functions of adipokines after SCI in generating metabolic and inflammatory changes. Even so, the present studies provide promising evidence that future endeavors in this area will prove worthwhile.

The changes of adipokine expression in the adipose tissue would have far reaching metabolic consequences, most notably in the liver as the primary metabolic organ. Previous studies have shown the chronic accumulation of lipids in the liver after thoracic spinal cord injury. Given that the Oil Red O stain used for this analysis is non-specific in its binding to lipid species, it was important to determine the lipid species that are altered after thoracic injury. Notably, ceramide content is increased in the liver following thoracic injury. Ceramide precursors (dihydroxyceramides), mature ceramide, and ceramide products (lactosylceramides) are all increased in the liver. Ceramides act as signaling molecules that act as second messengers in apoptosis signaling cascades, and their prolonged presence can lead to oxidative damage^{14,40}. These cytotoxic effects cause ceramides to exacerbate liver pathology and are a further indication of liver dysfunction.

Ceramides have also been shown to be important in the pathogenesis of NAFLD and progression to NASH^{27,33,38}. Their overexpression has been linked with insulin resistance, particularly in the liver, as well as furthering recruitment of inflammatory cells. This is even more concerning because of the rapid onset of these changes – as soon as 1 dpi. While some species – such as mature ceramide – return to baseline levels after the acute increase, other species remain elevated chronically to at least 42 dpi. The initial liver steatosis and inflammation is only enhanced by the chronic increase in ceramide content and may aid in progression of liver dysfunction to clinical relevance. Vitamin E levels increase in the liver following the same time course as changes to ceramide content. As a lipid soluble vitamin, the increasing levels of Vitamin E are further evidence of enhanced fat accumulation in the liver. Vitamin E is a potent antioxidant, and its presence can help to prevent the cytotoxic oxidative damage brought about by prolonged ceramide increase. Its increase is a result of increased hepatic lipid influx, but its antioxidant properties

would suit the post-injury fatty liver environment. It is also a potential therapeutic target as previous studies have shown its preventative effects in liver dysfunction³⁴.

Data shown in Fig 2.4 shows the origin of the ceramide increase. The up regulation of gene expression for SPT indicates that the source of increased liver ceramide content is the de novo synthesis from the liver itself. Excess ceramides are not generated from breakdown of the plasma membrane component sphingomyelin because nSMase expression is not significantly changed after injury. Ceramides act as a second messenger through programmed cell death, particularly through tumor necrosis factor signaling, mainly through TNF- α ²⁶. However, their appearance in properly functioning tissue is typically due to the breakdown of membranous sphingomyelin by nSMase^{3,14}. Thus the unchanged nSMase expression coupled with enhanced SPT expression is indicative of dysfunction. Inhibition of de novo ceramide synthesis enzymes has been shown to reduce lipid accumulation in NAFLD patients²¹. The increased SPT expression after injury gives promising evidence that this treatment could also be applied as a therapeutic measure to the SCI patient population to combat fatty liver pathology. A “multiple hit” pathophysiology is most accepted in the medical community for development of NAFLD and NASH, and elimination of increased ceramide expression would remove the “hits” of inflammation and insulin resistance involved in development of pathology.

Changes in cytokine expression are observed in development of NAFLD and NASH in the uninjured population, notably the increase in TNF- α expression^{7,45}. This same trend occurs in spinal cord injury, with significant increases developing in thoracic injury as soon as two weeks post injury. TNF- α is secreted directly by hepatocytes and in adipose tissue as a mediator of the inflammatory response, leading to its association with development of NASH¹⁶. The significant increase at 14 and 21 dpi is another marker of rapid pathological progression in the liver. TNF- α has several roles in mediating the inflammatory response, and one way it accomplishes this is through increasing ceramide synthesis by enhancing SPT expression⁶. This follows earlier observations that show increases in ceramides by the de novo synthesis pathway of which SPT is the rate-limiting enzyme. Also shown in Fig 2.5 (B) is the non-significant change in IL-6 expression. IL-6 plays a rather paradoxical role in liver dysfunction, and while it acts as a pro-inflammatory cytokine, it has also been shown to have protective action for hepatocytes³⁹. Despite trending towards increased expression, IL-6 is not significantly changed after injury. IL-10 has also been studied for its protective effects on hepatocytes against lipotoxicity from fatty liver pathology. Levels of IL-10 have been inversely correlated with metabolic syndrome, and can protect against further progression of NAFLD and NASH⁴⁵. IL-10 shows the least amount of change relative from naïve levels and, like IL-6, is not significantly changed after injury.

The increase in inflammatory TNF- α without the increase in protective IL-6 or IL-10 is evidence of further liver damage. Without anti-inflammatory effects of IL-10 or adiponectin, TNF- α and ceramides produce a self-proliferating cycle of inflammatory changes in the liver³³. Some evidence has shown anti-TNF- α treatment to be effective in ameliorating liver steatosis and inflammation, but these studies were performed in uninjured animals on a high-fat diet³⁵. It would be important to consider the ramifications of exploring this treatment option in patients reacting to a traumatic SCI. As TNF- α is involved in regulation of the immune response after injury, therapeutic targets for SCI-patients with NASH could best be targeted elsewhere to prevent attenuation of the innate response necessary for proper healing.

It is critical to remember that spinal cord injury to different levels of the cord could have different functional consequences for the regulation of the liver¹¹. Primary sympathetic afferents that directly control liver function are housed in the thoracic spinal cord. Damage to the thoracic region would damage these direct inputs to the liver, allowing it to act with only parasympathetic input. Damage to the cervical cord would result in damage to the descending control of the sympathetic nervous system, but would still allow the sympathetic neurons in the thoracic cord to act independently of higher CNS control. However, the data provides evidence that cervical injury produces comparable liver dysfunction as thoracic injury. Liver lipid content as shown in Fig 2.6 shows significant accumulation over the same time course as thoracic injury. Production of inflammatory cytokines IL-1 α and IL-1 β in the liver is also increased chronically in both injury models. These changes in liver function after cervical injury mimic the fatty and inflamed livers associated with thoracic injury. It can be concluded that both cervical and thoracic injury types result in a fatty and inflamed liver and produce comparable liver dysfunction.

One notable trend in both injury types is the pattern of lipid accumulation and inflammation after injury. After the initial increase in the acute phase (1-3 dpi), there is a return to naïve levels around 7 dpi and then a chronic increase that becomes significant at 21 dpi (see Figs 1.1, 1.2, 2.6). It is possible that this pattern is due to a prolongation of the acute phase response. Sham controls mimic the changes seen for the first few days after injury, but do not show the chronic increased observed in injured animals. Damage to the nervous system may impair a return to normal physiological function, and imbalances steadily become more pronounced after the acute phase response has attempted to return to normal function. The similar changes observed in the two injury groups indicate that the conclusions made from these studies would be applicable to a broad patient base, and more specific determinants would be necessary to differentiate between cervical and thoracic injury on liver dysfunction. Future studies may seek to examine if lumbar

level injuries cause a similar liver dysfunction, as a lumbar injury damages neither sympathetic output nor descending control of autonomic liver innervation.

Pathological changes in the liver occur after both cervical and thoracic SCI and are characterized by increased lipid accumulation including changes to ceramide composition, inflammation, and release of alanine transaminase. Notably, the array of post-SCI hepatic changes mimics the condition of non-alcoholic steatohepatitis, which is associated with insulin resistance, peripheral inflammation, and metabolic syndrome. This condition is concerning because it can progress to liver cirrhosis. The only accepted current treatment for NAFLD-like pathology is body weight loss, which is often not a viable option for SCI patients because of mobility limitations. Given the liver's essential role in metabolic function and the known metabolic problems after SCI, these studies serve as an important first step to identify novel therapeutic targets for improving health, longevity, and quality of life for SCI patients.

References

1. Aguilar, J., Humanes-Valera, D., Alonso-Calvino, E., Yague, J. G., Moxon, K. A., Oliviero, A., et al. (2010). Spinal cord injury immediately changes the state of the brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, 30(22), 7528-7537.
2. Almad, A., & McTigue, D. M. (2010). Chronic expression of PPAR- δ by oligodendrocyte lineage cells in the injured rat spinal cord. *The Journal of Comparative Neurology*, 518(6), 785-799.
3. Andrieu-Abadie, N., & Levade, T. (2002). Sphingomyelin hydrolysis during apoptosis. *Biochimica Et Biophysica Acta*, 1585(2-3), 126-134.
4. Bauman, W. A., & Spungen, A. M. (2001). Carbohydrate and lipid metabolism in chronic spinal cord injury. *The Journal of Spinal Cord Medicine*, 24(4), 266-277.
5. Benomar, Y., Gertler, A., De Lacy, P., Crepin, D., Ould Hamouda, H., Riffault, L., et al. (2013). Central resistin overexposure induces insulin resistance through toll-like receptor 4. *Diabetes*, 62(1), 102-114.
6. Braunersreuther V, Viviani GL, Mach F, Montecucco F., (2012). Role of cytokines and chemokines in non-alcoholic fatty liver disease. *World Journal of Gastroenterology : WJG*, 18(8), 727-35.
7. Crespo J, Cayón A, Fernández-Gil P, Hernández-Guerra M, Mayorga M, Domínguez-Díez A, Fernández-Escalante JC, Pons-Romero F., (2001). Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. *Hepatology (Baltimore, Md.)*, 34(6), 1158-63.
8. De Biase, A., Knobloch, S. M., Di Giovanni, S., Fan, C., Molon, A., Hoffman, E. P., et al. (2005). Gene expression profiling of experimental traumatic spinal cord injury as a function of distance from impact site and injury severity. *Physiological Genomics*, 22(3), 368-381.
9. Farrell, G. C., & Larter, C. Z. (2006). Nonalcoholic fatty liver disease: From steatosis to cirrhosis. *Hepatology (Baltimore, Md.)*, 43(2 Suppl 1), S99-S112.
10. Felipe V, . (20). Hepatic encephalopathy: Effects of liver failure on brain function. *Nature Reviews.Neuroscience*, 14(12), 851-8.
11. Fleming, J. C., Bailey, C. S., Hundt, H., Gurr, K. R., Bailey, S. I., Cepinskas, G., et al. (2012). Remote inflammatory response in liver is dependent on the segmental level of spinal cord injury. *The Journal of Trauma and Acute Care Surgery*, 72(5), 1194-201;discussion 1202.
12. Gabay C, K. I., (1999). Acute-phase proteins and other systemic responses to inflammation. *The New England Journal of Medicine*, 340(6), 448-54.
13. Gerrits, A. J., Gitz, E., Koekman, C. A., Visseren, F. L., van Haften, T. W., & Akkerman, J. W. (2012). Induction of insulin resistance by the adipokines resistin, leptin, plasminogen activator inhibitor-1 and retinol binding protein 4 in human megakaryocytes. *Haematologica*, 97(8), 1149-1157.
14. Haimovitz-Friedman, A., Kan, C.-C., Ehleiter, D., Persaud, R. S., (1994). Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *The Journal of Experimental Medicine*, 180(2), 525.

15. Holland, W. L., Miller, R. A., Wang, Z. V., Sun, K., Barth, B. M., Bui, H. H., et al. (2011). Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. *Nature Medicine*, 17(1), 55-63.
16. Hotamisligil, G. S., Shargill, N. S., & Spiegelman, B. M. (1993). Adipose expression of tumor necrosis factor- α : Direct role in obesity-linked insulin resistance. *Science (New York, N.Y.)*, 259(5091), 87-91.
17. Ibrahim, M. A., Kelleni, M., & Geddawy, A. (2013). Nonalcoholic fatty liver disease: Current and potential therapies. *Life Sciences*, 92(2), 114-118.
18. Inskip J, Plunet W, Ramer L, Ramsey JB, Yung A, Kozlowski P, Ramer M, Krassioukov A., (2010). Cardiometabolic risk factors in experimental spinal cord injury. *Journal of Neurotrauma*, 27(1), 275-85.
19. Kaser, S, Kaser, A, Sandhofer, A, Ebenbichler, C.F, Tilg, H, Patsch, J.R., (2003). Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. *Biochemical and Biophysical Research Communications Biochemical and Biophysical Research Communications*, 309(2), 286-290.
20. Koj, A., (1996). Initiation of acute phase response and synthesis of cytokines. *Biochimica Et Biophysica Acta*, 1317(2), 84-94.
21. Kurek, K., Piotrowska, D. M., Wiesiolek-Kurek, P., Lukaszuk, B., Chabowski, A., Gorski, J., et al. (2013). Inhibition of ceramide de novo synthesis reduces liver lipid accumulation in rats with nonalcoholic fatty liver disease. *Liver International : Official Journal of the International Association for the Study of the Liver*,
22. Lafontan, M., (2014). Adipose tissue and adipocyte dysregulation. *Diabetes & Metabolism Diabetes & Metabolism*, 40(1), 16-28.
23. Lalor PF, Faint J, Aarbodem Y, Hubscher SG, Adams DH., (2007). The role of cytokines and chemokines in the development of steatohepatitis. *Seminars in Liver Disease*, 27(2), 173-93.
24. Lavela, S. L., Weaver, F. M., Goldstein, B., Chen, K., Miskevics, S., Rajan, S., et al. (2006). Diabetes mellitus in individuals with spinal cord injury or disorder. *The Journal of Spinal Cord Medicine*, 29(4), 387-395.
25. Listenberger, L. L., Han, X., Lewis, S. E., Cases, S., Farese, R. V., Ory, D. S., et al. (2003). Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proceedings of the National Academy of Sciences*, 100(6), 3077-3082.
26. Liu, H., Wu, X., Dong, Z., Luo, Z., Zhao, Z., Xu, Y., et al. (2013). Fatty acid synthase causes drug resistance by inhibiting TNF- α and ceramide production. *Journal of Lipid Research*, 54(3), 776-785.
27. Longato L, Tong M, Wands JR, de la Monte SM., (2012). High fat diet induced hepatic steatosis and insulin resistance: Role of dysregulated ceramide metabolism. *Hepatology Research : The Official Journal of the Japan Society of Hepatology*, 42(4), 412-27.
28. Luo, R., Li, X., Jiang, R., Gao, X., Lu, Z., & Hua, W. (2012). Serum concentrations of resistin and adiponectin and their relationship to insulin resistance in subjects with impaired glucose tolerance. *The Journal of International Medical Research*, 40(2), 621-630.

29. Malaguarnera, M., Di Rosa, M., Nicoletti, F., & Malaguarnera, L. (2009). Molecular mechanisms involved in NAFLD progression. *Journal of Molecular Medicine (Berlin, Germany)*, 87(7), 679-695.
30. Montecucco, F., & Mach, F. (2008). Does non-alcoholic fatty liver disease (NAFLD) increase cardiovascular risk? *Endocrine, Metabolic & Immune Disorders Drug Targets*, 8(4), 301-307.
31. National Spinal Cord Injury Statistical Center,. (2013). Spinal cord injury facts and figures at a glance. *The Journal of Spinal Cord Medicine*, 36(1), 1-2.
32. Ouchi, N., Parker, J. L., Lugus, J. J., & Walsh, K. (2011). Adipokines in inflammation and metabolic disease. *Nat Rev Immunol*, 11(2), 85-97.
33. Pagadala, M., Kasumov, T., McCullough, A. J., Zein, N. N., & Kirwan, J. P. (2012). Role of ceramides in nonalcoholic fatty liver disease. *Trends in Endocrinology and Metabolism: TEM*, 23(8), 365-371.
34. Patel V, Rink C, Gordillo GM, Khanna S, Gnyawali U, Roy S, Shneker B, Ganesh K, Phillips G, More JL, Sarkar A, Kirkpatrick R, Elkhammas EA, Klatte E, Miller M, Firstenberg MS, Chiocca EA, Nesaretnam K, Sen CK,. (2012). Oral tocotrienols are transported to human tissues and delay the progression of the model for end-stage liver disease score in patients. *The Journal of Nutrition*, 142(3), 513-9.
35. Pinto Lde, F., Compri, C. M., Fornari, J. V., Bartchewsky, W., Cintra, D. E., Trevisan, M., et al. (2010). The immunosuppressant drug, thalidomide, improves hepatic alterations induced by a high-fat diet in mice. *Liver International : Official Journal of the International Association for the Study of the Liver*, 30(4), 603-610.
36. Schmittgen, Thomas D., Livak, Kenneth J.,. (2008). Analyzing real-time PCR data by the comparative [C.sub.T] method.(PROTOCOL)(polymerase chain reaction). *Nature Protocols*, 3(6)
37. Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA,. (2005). Activation of SOCS-3 by resistin. *Molecular and Cellular Biology*, 25(4), 1569-75.
38. Summers, S. A. (2006). Ceramides in insulin resistance and lipotoxicity. *Progress in Lipid Research*, 45(1), 42-72.
39. Tilg, H... (2010). The role of cytokines in non-alcoholic fatty liver disease. *Digestive Diseases*, 28(1), 179-185.
40. Won, Je-Seong, Singh, Avtar K., Singh, Inderjit,. (2007). Lactosylceramide: A lipid second messenger in neuroinflammatory disease. *Journal of Neurochemistry*, 103(Supplement), 180-191.
41. Yang, Guang, Badeanlou, Leylla, Jacek Bielawski, Roberts, Amanda J., Hannun, Yusuf A., Samad, Fahumiya,. (2009). Central role of ceramide biosynthesis in body weight regulation, energy metabolism, and the metabolic syndrome. *American Journal of Physiology: Endocrinology & Metabolism*, 60(1)
42. Yang, S. Q., Mandal, A. K., Huang, J., & Diehl, A. M. (2001). Disrupted signaling and inhibited regeneration in obese mice with fatty livers: Implications for nonalcoholic fatty liver disease pathophysiology. *Hepatology*, 34(4), 694-706.
43. Yi, C., la Fleur, S. E., Fliers, E., & Kalsbeek, A. (2010). The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1802(4), 416-431.

44. Yuan, Y. M., & He, C. (2013). The glial scar in spinal cord injury and repair. *Neuroscience Bulletin*, 29(4), 421-435.
45. Zahran W.E., Salah El-Dien K.A., Kamel P.G., El-Sawaby A.S., (2013). Efficacy of tumor necrosis factor and interleukin-10 analysis in the follow-up of nonalcoholic fatty liver disease progression. *Indian J.Clin.Biochem.Indian Journal of Clinical Biochemistry*, 28(2), 141-146.

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